429. MICROBIAL RENNET SUBSTITUTES—A REVIEW

I. J. BABBAR, R. A. SRINIVASAN, S. C. CHAKRAVORTY AND A. T. DUDANI
National Dairy Research Institute, Karnal

The enzyme rennet is almost universally employed for the production of different varieties of cheeses, although pepsin and a commercial product under the trade name of Metroclot (Maragondakis *et al.*, 1961; Melachouris & Tuckey, 1964) have also been used for the purpose. Attempts have been made from time to time to find a suitable substitute for animal rennet. The chief factors that have prompted investigations on this subject are the non-acceptability of these products of animal origin to vegetarian population particularly in countries like India and Israel, its present acute shortage in major rennet-producing countries and its non-availability at competitive prices.

In India, there has been perceptible increase in the demand for cheese, although no precise information is available with regard to the amount of enzyme required by the Dairy industry for this purpose. There is thus considerable potential in this direction, particularly, in this country, if a suitable milk clotting enzyme of non-animal origin (plant or microbial) could be made available.

Coagulating Enzymes from Plants

Some of the plant materials studied and reported in the literature include those of genus *Ficus* such as *Ficus glomarata, Ficus religiosa* and in particular *Ficus carica* (Krishnamurthy & Subrahmanyan, 1948, 1949; Whitaker, 1959; Zukerman, Stark & Leibowitz, 1961, 1963; Krishnaswamy *et al.*, 1961), *Withania coagulans* (Dastur, Sastry & Venkatappiah, 1948; Kothavalla and Khubchandani, 1940; Narain & Singh, 1942), *Carica papaya* (Dastur, 1949); papain (Windland & Kosikowsky, 1946), *Cynara* species (Russo, 1961; Christian & Vierasoro, 1935; Pereira & Viereira, 1948) and pumpkin (Rebecca & Leibowitz, 1963). Although no detailed information is available in respect of the quality of cheese prepared using these enzymes but in general the cheeses made appear to develop bitter taste in varying degrees, bitterness decreasing in 3 to 6 months (Krishnaswamy *et al.*, 1961).

Rennet Substitutes from Micro-organisms

A large number of micro-organisms are known to produce milk clotting enzymes. The possibility of a few of the micro-organisms proving successful as rennet substitutes may appear from the fact that they play an important part during the manufacture of cheese. Microbial enzymes are known to exhibit considerable variations in the range of activity, substrate specificity and mode of action. Even more important is the fact that they can be produced economically on any desired scale. The reports on the suitability of various microbial enzymes for cheese manufacture are, however, very scanty and compared to the plant sources, interest in them is comparatively of recent origin. The crude preparations exhibiting milk clotting activities are invariably accompanied by proteolytic enzymes. It is known that coagulation of milk with such enzyme preparations leads to precipitation of the colloidal milk casein as calcium paracaseinate and a glycopeptide is released from K-casein fraction of the molecule (Whitaker, 1959). This precipitated material undergoes various primary and secondary biochemical changes in which proteolytic enzymes play an important part. The micro-organisms known to produce appreciable amounts of such enzymes are listed in Table 1. As indicated, some of these have been employed as rennet substitutes with varying degree of success. Micro-organisms other than those which have yielded rennet substitutes have also been presented in the table for comparative purpose with a view to briefly outline the conditions for the production of enzymes of various cultures which may possibly be of value as potential sources of coagulating enzyme.

It will be seen from Table 1 that the media constituents employed for the production of the enzyme were of two types:

(*a*) synthetic media which contained the protein sources such as casein, thin-milk, gelatin, zein, etc., and carbohydrate sources such as glucose, lactose or starch, etc.;

(*b*) natural media which was composed of cereal brans, oilseed cakes such as peanut cake, malt extract, yeast extract, soyabean, skim-milk, etc.

Usually the enzyme activities elaborated by various micro-organisms have been found to be

TABLE 1. *Production of Milk Clotting Enzymes*

Micro-organisms	Medium and other conditions of production	Reference	Nature of cheese if prepared
1	2	3	4
	A. *Bacteria*		
Bacillus brevis ..	..	Shimwell and Evans (1944)	Several varieties of cheese made but no details given
B. cereus ..	Skim-milk and papain digested skim-milk	Srinivasan *et al.* (1962)	Cheddar cheese obtained was good with no bitter taste
B. coagulans ..	Skim-milk	Varma *et al.* (1951)	..
B. licheniformis ..	Peanut cake and glucose	Damodaran *et al.* (1955)	..
B. mesentericus ..	Potato, wheat bran, barley, oats and calcium carbonate	Emanuiloff (1956)	Cheese obtained comparable to rennet
..	..	do. (1959)	..
B. subtilis ..	Skim-milk and papain digested skim-milk	Srinivasan *et al.* (1962)	As in *B. cereus*
do. ..	..	Shimwell and Evans (1944)	As in *B. brevis*
do. ..	Starch, zein, casein	Smythe *et al.* (1950)	..
do. ..	Carbohydrate 6%, protein, such as soyabean, peanut cake, 0·75–1%	Kline (1944)	..
do. ..	Malt extract and yeast extract	Wallerstein (1937)	..
do. ..	Cereal brans or their extracts and oil-seed cakes	Feniksova and Tikhomirova (1960)	..
do. ..	Cereal brans or their extracts *plus* oil-seed cakes	Babbar *et al.* (1961)	..
Pseudomonas fluorescens	Low temperature	Peterson and Gunderson (1960)	..
Ps. myxogenes	Glucose 7%, corn steep liquor 0·125%, calcium carbonate	Morihara (1957)	..
		Castaneda (1956)	..
		Virtanen (1950)	..
Streptococcus liquefaciens	Skim-milk and lactose	Srinivasan *et al.* (1962)	Satisfactory cheese
do.	..	Srinivasan (1964)	..
do.	..	Rabin+Zimmerman (1956)	..
S. liquefaciens	Skim-milk casein	Dudani (1950)	..
Streptococcus zymogenes	..	McDonald (1958)	..
Bacillus sp. ..	Yield acid stable enzyme	Trione (1960)	..
do. ..	Milk giving acid endopeptidase	Babbar *et al.* (1964)	..
Spore-forming bacteria	..	Kikawa (1959)	Bitter peptides produced from casein
do. ..	Whole milk	Srinivasan *et al.* (1964)	..

considerably higher when natural media are employed than in synthetic media. The stimulation of the enzyme production in natural media may be ascribed to the presence of not only good protein and carbohydrate source but also various undefined growth factors like vitamins, calcium phytate, etc. The stimulatory effect of calcium phytate on the production of milk clotting and proteolytic enzymes of spore-forming bacteria have been reported recently (Srinivasan *et al.*,

Micro-organisms	Medium and other conditions of production	Reference	Nature of cheese if prepared
1	2	3	4
	B. *Molds*		
Asp. flavus oryzæ ..	Semi-solid bran	Oshima and Church (1923)	..
	do.	Wallerstein (1937)	..
	do.	Waksman (1927)	..
	do.	Tanaka *et al.* (1960)	..
	Raulin's medium (surface) —(submerged)	Maxwell (1952)	..
		Motonage (1960)	..
A. nidulans ..	UVL	Brockaia (1960)	..
P. chrysogenum ..	Peanut cake and sucrose or lactose	Singh and Ghosh (1958)	..
do. ..	Autolysate of Mycelia	Besboradova (1960)	..
Entomophthorale sp. ..	..	Oringer (1960)	Good cheese obtained
	..	White hill *et al.* (1960)	do.
	..	French Pat (1960)	Cheese satisfactory
	C. *Actinomycetes*		
Streptomyces griseus	Medium as used for streptomycin production	Ouchi (1962) and Nomoto (1959)	..
		Watanabe *et al.* (1959)	..
do.	Gelatin	Dion (1950)	..
	Protein nitrogen gives least production, peptides and amino-acids gave more enzyme	Chaloupka (1956)	..
		do. (1958)	..

1964; Dunn *et al.*, 1959; Tsuchihira, 1959). The natural media have also the added advantage of being economical and readily available in India.

Micro-organisms found as potent sources of renneting activity have been confined to spore-forming bacteria and a few species of *Pseudomonas* (*loc. cit.*) and among the fungi *Entomophthorales* (*loc. cit.*) the latter having come into the picture only recently. Cheese of satisfactory quality has been reported to have been made from these micro-organisms. There thus appears to be considerable scope for extension of work on these organisms.

Production of Microbial Milk Clotting Enzymes

Various factors that must be taken into consideration for the production of these enzymes are :

(*i*) Screening of micro-organisms—a large number of microbes of various species should be isolated from sources like soil, milk and milk products including butter, cheese and cream, etc. A few potent strains are then selected for further work.

(*ii*) Cultural conditions—for maximum enzyme production, media constituents, pH of media, temperature and mode of growth of micro-organisms, period of growth, etc., have to be investigated.

(*iii*) Recovery of enzyme—usually protein precipitants such as ammonium sulphate, organic solvents like ethyl alcohol, acetone, etc., are employed for this purpose at low temperatures. The precipitated enzyme is usually dried preferably *in vacuo* at low temperatures to avoid loss in activity of the enzyme.

It is also customary to standardise commercial preparations to activities which can be employed directly in the manufacturing plants.

Processes employed for the cultivation of the selected microbes for the production of milk clotting enzymes are in general similar to those for proteolytic enzymes:

(*a*) *Bran Process.*—In this process moistened cereal brans (usually wheat bran) are cooked under pressure to gelatinise starch and sterilise the mash, cooled to desired temperature and inoculated with the selected organism. The inoculated medium may be allowed to grow in shallow trays in thin layers (Shellenberger, 1947; Underkofler *et al.,* 1951) or in rotating drums (Underkofler *et al.,* 1939), the latter method has generally yielded place to the shallow tray method (Lockwood, 1952). The enzyme is then extracted from the bran using suitable solvent precipitants, adsorption techniques, etc. Bran process is generally employed for the growth of molds.

(*b*) *Liquid Surface Process.*—The organism is allowed to grow on the surface of the liquid media, which may be 'synthetic' or 'natural'. The chief drawback of this method is that large amount of space, labour and equipment is required; the maintenance of sterile conditions and temperature control on a commercial scale plant also offers considerable difficulties.

(*c*) *Submerged Process.*—In this process the micro-organisms are cultivated in the entire mass of the liquid media by the application of vigorous aeration and agitation. This method has mainly replaced the semi-solid or the liquid surface processes because of the advantages it enjoys over these, namely, great economy in equipment, labour and space. This process has been extensively employed for the production of proteolytic and other enzymes of commercial interest. While some recent reports about the effect of shaking on the production of enzymes appear to be contradictory (Richon and Kourilsky, 1959; Morihara, 1957), but on the whole vigorous aeration and agitation have favourable effect on high production of these enzymes. In general, calcium has been found to exert a favourable influence on the production and stabilisation of microbial proteases (Emanuiloff, 1956, 1959; Haines, 1931, 1933; Merrill & Clark, 1928; Gorini & Fromageot, 1950; Castaneda, 1956). In this connection, the production of milk clotting enzymes by continuous cultivation using Bactogen (Monod, 1950) or Chemostat (Novick, 1958) type of equipment may also offer some possibilities.

Methods of Assay

Many methods for estimating the potency of milk-clotting enzymes have been described in literature. Among the earlier methods those referred to in Oppenheimer's *Fermente und Ihre Wirkungen* (1929) and Rona (1931) may be mentioned in passing although these are no longer in vogue. In most of these methods the activity of the milk-clotting enzyme is determined by measuring the time required to clot a suitable substrate (fresh milk or skim-milk powder solution) under defined conditions. Special casein preparations have also been used (Salati, 1953). The amount of the enzyme added must be so chosen that the clotting time falls in the range in which it is inversely proportional to the concentration of the enzyme.

Kunitz (1935) employed Klim (milk powder) solution (18%) at pH 5·0 (acetate buffer, 0·1M) to which 1 ml. of the enzyme of suitable dilution at temperature of 35·5° C was added and the time of clot measured. One unit of the enzyme activity was expressed as the amount of enzyme that causes clotting of the milk solution in one minute so that if t minutes were required to clot the milk by 1 ml. of the enzyme, the enzyme potency was expressed as $1/t$ units of rennet activity/ml. The specific activity of the enzyme per mg. protein nitrogen was indicated as (T.u) rennet $= 1/t \times$ mg. protein nitrogen per ml.

In the method of Balls and Hoover (1937), to 10 ml. of 20% milk powder solution at pH 4·6 acetate buffer (0·1 M) was added 1 ml. of the enzyme at 40° C. The time of clot is usually kept between 1 and 5 minutes. Except for small amount of the enzyme the time of clot is inversely proportional to the amount of enzyme present. When E is the weight of the enzyme (in mg.) and t is time of clot (in minutes) then $E = k/t$ or $E \times t = k$ is, therefore, the enzyme units. A part of the enzyme is invariably inhibited by milk and the modified equation is $(E - c)\ t = k$, where c represents the amount of inhibited enzyme. This inhibition of the enzyme can be reduced considerably by employing lesser amount of the substrate (Whitaker, 1959).

Tauber (1949) used fresh cow's milk buffered to pH 5·0 at 20° and added graded amounts of the enzyme under investigation in various test-tubes. The tube-clotting milk in 10 minutes is noted. The rennet activity equals mg. of enzyme required to clot 10,000 mg. of milk.

The method of Berridge (1952) requires the formation under reproducible conditions of a thin flowing film of milk. A test-tube containing the rennetic milk is rotated in a water-bath in which it is set at an angle of 30° and the end point is observed when clotting milk forms a thin opaque film on the walls of the tube. The method requires a special apparatus.

Although these methods employed animal rennet as source of the enzyme, these can also be adopted for assay of the enzyme derived from micro-organisms. In the hands of the present authors the method of Balls and Hoover (*loc. cit.*) has been found to be satisfactory. If milk powder is not available, fresh pasteurised cow milk (pasteurised at 70° C for 1 minute) to which calcium chloride in the concentration of 0·01 M is added may be employed as substrate (Srinivasan *et al.*, 1964). Calcium chloride prevents to an appreciable extent the changes of calcium phosphate between ionised and colloidal solution (Payne, 1945, 1948). The presence of calcium chloride also helps to reduce the time lag between the change in the substrate and the clotting time. In fact, purified calcium-free casein dissolved in citrate buffer (Lundsteen, 1938) would be theoretically more profitable since many factors which affect the sensitivity of milk to the enzyme are thus absent. The pasteurised milk can be kept in cold under layer of toluene and used for the assay for 2–3 days.

Viscometric methods have also been suggested recently by Scott Blair *et al.* (1963) and Oosthuizen (1962, 1963) for the assay of the enzyme using 2 per cent and 6 per cent sodium caseinate solutions. Scott Blair and co-workers (*loc. cit.*) used a simple apparatus devised by them for this purpose in which the height of the milk column was measured with time and *tc* which is time when points on this curve start to diverge from straight line is determined. These workers have compared the *tc* obtained by them and that obtained in Berridge's method and found the latter came a few minutes later. Oosthuizen (*loc. cit.*) has reported the activities of vegetable rennets derived from *Ficus carica* and *Withania coagulans* by viscometric method. The viscometric methods have not been used extensively yet probably because they may represent more a reflection of the proteolytic component of the enzyme complex rather than the milk-clotting enzyme.

Discussion and Conclusion

Investigations reported so far have shown that the microbial rennet substitutes usually found satisfactory are those derived from spore-forming bacteria, with *Pseudomonas* species being an exception. It would, therefore, seem that spore-forming organisms offer the best avenues and maximum attention is required to be paid to this class of bacteria.

It is also likely that the characteristics of rennet substitute from one microbial source may be improved considerably by blending them with preparations from other sources such as those derived from fungi or possibly actinomycetes.

The main criteria for a good rennet substitute proposed by Veringa (1961) and Tsugo and Yamamuchi (1959) are that the enzyme substitute should (*i*) coagulate milk in temperature range of 25° C to 45° C, (*ii*) give satisfactory yield of cheese, (*iii*) low loss of fat, (*iv*) yield cheese having desired texture and other physical properties, (*v*) promote correct changes during ripening and (*vi*) impart characteristic flavour and taste (organoleptic tests). Another important consideration which can be added to this would be early ripening of cheese. In order to plan up a programme of work on rennet substitutes, it would be profitable to revolve around the above considerations as basis of the investigation. This would involve the isolation of the enzyme, partial purification and actual trials on the manufacture of cheese followed by proper curing of the cheese from 3–12 months. This would seem a stupendous job when one has to investigate large number of enzyme sources for ascertaining their suitability.

It is known that stage of milk-clotting involves the release of glycopeptide from K-casein and subsequent precipitation of casein as calcium α-paracaseinate (*loc. cit.*). In cheese the clot thus formed is allowed to undergo further changes during curing involving primary and secondary stages, particularly, proteolysis. The proteolytic changes are brought about by the proteolytic enzymes present in rennet used for cheese manufacture, in starter cultures and also secreted by the microflora which develop on the cheese during the curing period. It is due to this important consideration in screening programme that the extent of the production of proteolytic enzymes should be followed in addition to milk clotting.

Srinivasan *et al.* have reported (1964) that the different isolates exhibit different ratios of milk clotting and proteolytic activities. This ratio was termed as Index of the isolate. It was suggested by these workers that in all probability milk clotting and proteolytic activities are due to two different enzymes, confirming through a different approach, the observations of Bargoin (1963) on the separate identity of the two enzymes. This Index may be of significant value in the screening of micro-organisms for this purpose. Isolates exhibiting indices near that of animal rennet are likely to yield enzyme preparation from which cheddar cheese of acceptable quality may be prepared.

Partial separation of the proteolytic activity from milk-clotting activity would help in bring-

ing about the desirable extent of proteolysis of casein during ripening. Whereas attempts at such separations have met with considerable success in enzyme systems from plant sources such as papaya latex (Jensen & Balls, 1941; Balls & Lineweaver, 1939) and fig latex (Krishnamurthy & Subrahmanyan, 1953; Whitaker, 1959) the two activities seemed to be inseparable in micro-organisms like *Streptococcus zymogenes* (Dudani, 1950; Frederick & Zimmerman, 1955) and *Coccus* P. (Gorini & Lanzavecchia, 1954).

Fundamental considerations might also help in elucidating the problem, *e.g.*, rennin belongs to the class of serine proteases (Hartley, 1960; Smith, Light & Kimmel, 1962), in which serine is the active centre of the enzyme. Ficin and papain which have been mentioned in the literature for cheese making, on the other hand, belong to the thiol class in which the presence of a sulphydryl group is essential as active centre. The latter enzymes usually have given bitter cheese. Serine enzymes are inactivated by reagents like di-isopropylphosphofluoridate (DFP) while thiol class is inactivated by alkylating agents or heavy metals and their compounds, particularly, *p*-chloromercuribenzoate.

If the above-mentioned factors are taken into consideration, it may be possible to work out a good substitute for rennet.

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